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Mouse mammary cell lines ranging from hyperplasia to highly tumorigenic were used to study distinct p53 protein forms during carcinogenesis. All contained p53 and an endogenous alternatively spliced form, p53as. p53/p53as mutation was neither sufficient nor required for the low tumorigenic phenotype, but mutated p53 accompanied high tumorigenicity. Genistein induced p53 and p53as (mutant or wild type) early, peaking 3 hours post treatment, and was transient, in contrast to the sustained induction of p53as in epidermal and fibroblast cells. Deficiency in sustained p53 response may diminish long-term p53 effects in apoptosis, differentiation or cell cycle arrest. The rapid induction of p53 and p53as shown by indirect immunofluorescence and immunoblotting was correlated with sequence-specific DNA binding in TM10 but not in TM3 mammary lines. Novel DNA motifs (TTGGC, ACTTG) in p53/p53as binding sequences retrieved by cyclic amplification and selection of targets (CASTing) were not sufficient to bind p53 or p53as, suggesting that one or more CATG is also required. A 20-bp homologue of the rat *mdr1* gene promoter retrieved by CASTing bound both p53 and p53as and may mediate p53 regulation reported for this gene. In order to test the hypothesis that the variability in induction and/or activity of wild type or mutant p53 proteins depends upon p53-associated proteins, mammary nuclear extracts were subjected to binding to glutathione-S-transferase/p53 or /p53as fusion proteins. Potential mammary-specific proteins are being isolated for microsequencing.

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INTRODUCTION

Mutation of the p53 tumor suppressor gene is the most common genetic defect in human cancers including breast cancer (Hollstein et al., 1994). p53 mutations are also found in Li-Fraumeni syndrome, a condition resulting in a predisposition to breast cancer development (Srivastava et al., 1990; Malkin et al., 1990). These facts suggest that the p53 protein may be a key component in maintaining mammary cell homeostasis and that determination of the regulation of expression and the functions of p53 in addition to identification of interacting proteins is of interest and importance in elucidating the mechanisms culminating in breast cancer.

The p53 protein has multiple functional domains and can exist in multiple forms (Wu et al., 1997). This laboratory has identified an alternatively spliced p53 mRNA and protein in normal mouse cells and tissues (Kulesz-Martin et al, 1994; Han and Kulesz-Martin, 1992) and is using it to analyze distinct p53 activities in response to DNA damage. The alternatively spliced protein, p53as, contains an altered C-terminus which is 9 amino acids shorter and contains 17 different amino acids than p53. Both p53as and p53 function as suppressors of growth (Wu et al., 1997) and both proteins bind the same DNA sequences (Miner and Kulesz-Martin, 1997). However, as a result of the change in the C-terminus, p53as exhibits certain distinct characteristics compared to p53. For example, p53as is preferentially associated with the G2 phase of the cell cycle while p53 is detectable in both G1 and G2 (Kulesz-Martin et al., 1994). p53as protein is constitutively active for DNA binding whereas p53 is frequently in a latent form which requires activation in order for DNA binding to be detectable (Wu et al., 1994). Finally, p53as and p53 were found to respond to DNA damage with different kinetics (Wu et al., 1997), suggesting that the multiple functions of p53 can be distinctly regulated.

To begin to examine the role(s) p53 and p53as play in mammary carcinogenesis and the progression of mammary cancer, a murine breast cancer model (Medina et al., 1993a; Medina et al., 1993b) was utilized in which both proteins could be studied at various stages of preneoplasia and malignancy. The Specific Aims of this proposal were: 1) to examine the expression of p53 and p53as during mammary cancer development, 2) to determine the half life of p53 and p53as

proteins and to detect associated proteins, and 3) to determine the oligomerization properties and DNA binding sequence specificity of p53as.

EXPERIMENTAL METHODS, RESULTS, AND DISCUSSION

TASK 1 (Aim 1) Determination of p53 and p53as expression in the mouse mammary model. The mouse mammary epithelial cell lines have been transplanted into syngenic mouse fat pads and shown to produce mammary preneoplastic outgrowths (Medina et al., 1993a). The "tumor mammary" (TM) established cell lines have been derived from the tumor outgrowths (Medina et al., 1993b). Table 1 shows the genotype and tumor phenotype of each TM line that was examined for expression of p53 and p53as.

As previously reported, RT-PCR analyses indicated that all of the TM lines expressed mRNA for both p53 and p53as and immunofluorescence studies determined that all lines expressed p53 and p53as proteins. Immunohistochemistry analysis of two TM tumors (3 and 10) which were representative of hyperplasia and low tumorigenicity, respectively, also expressed both p53 and p53as proteins.

In the 1995 progress report, we described preliminary studies of induction of p53 and p53as by DNA damage with actinomycin D at 24 or 48 hours following treatment and of p53/p53as induction in one cell line (TM10) after treatment with genistein. These experiments were carried out to determine the feasibility of using one of these agents to enhance visualization of p53 and p53as for the protein stability investigations, since the p53 levels detectable by immunoprecipitation were too low to permit the half-life studies in these cells. These agents have been shown to induce the expression of p53 and p53as in other cell lines previously (Kastan et al., 1991; Wu et al., 1997). Actinomycin D did increase expression of p53 and p53as antigen activity in TM3 and genistein induced expression of p53 and p53as in TM10.

Recently we reported that p53 and p53as in epidermal cells are induced with different kinetics (Wu et al., 1997). Latent forms for sequence-specific DNA binding (p53 homo-oligomers) are induced rapidly and transiently, while active forms (p53as homo-oligomers) are

sustained up to 48 hours after treatment with genistein. This year we report an extended time study of p53 and p53as induction in TM cells after treatment with genistein. Proliferating cells were treated with concentrations of genistein (20-30 μM) so as to achieve an IC_{50} then isolated at 0, 3, 16, and 24 hours following treatment for indirect immunofluorescence. All TM lines examined contain p53 and p53as. In addition, conformation-specific antibodies PAb246 and PAb240 were used to determine wildtype or mutant conformation proteins respectively. TM9, 10, and 12 have wildtype p53/p53as and TM4 has mutant proteins. Surprisingly, the proteins in TM3 did not react with either PAb246 or 240. The response of the TM cells lines to genistein treatment indicates transient induction of p53/p53as that occurs 3 hours following drug treatment. Baseline levels of both proteins are achieved by 24 hours post treatment.

In order to test function of the p53 proteins in TM cells, whole cell extracts from cells untreated or treated with genistein for 0, 3, 16, 24,and 48 hours were used in DNA binding assays with a labeled p53/p53as-binding DNA probe. These assays indicate that the p53/p53as proteins present in TM4, TM10, and TM12 bind DNA, but that the proteins present in TM3 and TM9 do not bind DNA. Thus, wild type p53 and p53as proteins are able to bind DNA in TM10 but not in TM9 cells, while mutant p53 protein is able to bind DNA in TM4 but not in TM3 cells. Reasons for this cell specificity are as yet unknown. However, these data lead to the hypothesis that cell type specific p53-associated proteins regulate p53 for DNA binding, which is the subject of Task 6 below. Representative data of TM10 and 4 (both binding) or TM3 (non-binding) cell lysates are shown in Figures 1, 2, and 3. Figure 1 shows assays utilizing lysates from TM10 cells. Both treated and untreated cells contain p53 and p53as binding activity. These activities appear to be induced by genistein treatment, peaking at 3 hours. The pattern of bands is similar to what has been observed using epidermal cell lysates (Wu et al., 1997) and, as indicated in figure 1, include latent p53 homo-oligomer (1), latent p53/p53as hetero-oligomer (2), active p53as homo-oligomer (3), and active p53/p53as hetero-oligomer. Quantification by phosphorimaging indicates that peak binding activity occurs at the 3-hour timepoint and is approximately two fold basal levels (time 0). A band (5) that migrates at a slightly lower molecular weight than latent

p53 homo-oligomers is present in the absence of PAb421 and is unaffected by the addition of ApAs at 3 and 16 hours of genistein treatment. This band migrates to a position different than active p53/p53as hetero-oligomers and is currently under investigation using multiple p53 antibodies to determine whether it is a complex containing p53. Consistent with its slower mobility, it is conceivable that the complex contains a distinct p53 family member such as the newly discovered p73 (Jost et al., 1997; Kaghad et al., 1997), which will be proposed for investigation in the future.

Figure 2 shows the results of binding assays using lysates from TM4 which contain latent p53 homo-oligomers (1) but lack latent p53/p53as hetero-oligomers (2), active p53as homo-oligomers (3) and active p53/p53as hetero-oligomers (4). Nonspecific bands that are unaffected by the addition of antibodies are seen in all lanes containing TM4 lysates. Quantitation of these data by phosphorimaging supports the impression by visualization that genistein does not induce increased expression of p53 in TM4 cells.

Using cell lysates from genistein-treated and untreated TM3 cells (Figure 3), there are no shifted bands in the presence or absence of antibodies PAb421 and/or ApAs migrating to similar positions as controls containing purified p53 or p53as protein ("controls"). The positions of p53as and p53 shifted bands in these controls are indicated. The lower molecular weight band seen in all lanes containing TM3 lysate is nonspecific as it is not supershifted by the addition of antibodies.

Western immunoblot analyses of the same lysates used in the above DNA binding assays indicate that TM10, 4, and 3 contain p53 and p53as (Figures 4, 5, and 6). These results are interesting in that although both TM3 and TM4 contain mutations in conserved regions of p53 (Region IV inTM3, Region II in TM4), TM4 but not TM3 mutant proteins bind DNA. These differences in binding may be a result of the types of mutations present in the proteins from the two cell lines (Table 1) or may be due to the presence of different p53-associated proteins present in TM4 that are able to convey a p53 protein conformation amenable to DNA binding. The affinity for binding by the TM4 mutant protein may not be as strong as the wildtype protein in

TM10 cells since cell lysates from both lines contain similar amounts of protein by western blot, but TM10 lysates give a stronger signal in DNA binding assays. As in the DNA binding assays, densitometric analysis of TM10 western immunoblotting indicates a 2-fold induction of p53 and p53as proteins after 3 hours of genistein treatment. TM3 and TM4 western immunoblots do not indicate an induction by genistein.

TASK 2 (Aim 2, 3) Production and purification of p53as and p53 proteins in the baculovirus system. This task was undertaken so that purified p53 and p53as proteins could be used to determine the oligomerization properties of p53as (Task 3) and as an alternative method to identify p53-associated proteins (Task 6). The oligomerization properties of p53 and p53as were determined using *in vitro* translated proteins, and p53-associated proteins have been observed using GST-fusion proteins. However, as reported last year, purified baculovirus-produced recombinant p53 and p53as proteins have been produced and are being used for controls in gel shift assays and are available for future studies of p53 associated proteins (Task 6) as an alternative approach to GST/p53 fusion proteins.

TASK 3 (Aim 2, 3) Determination of oligomerization properties of p53as. This task was completed as detailed in the first year progress report. Both p53 and p53as form homo- and hetero-dimers and tetramers (Wu et al., 1994).

TASK 4 (Aim 2) Determination of the half-life of p53as and p53 proteins. As reported in the 1995 progress report, the half life of p53 and p53as was determined in asynchronous TM3 and TM4 cells to be approximately 5 hours for p53 and 6 hours for p53as. This is consistent with the fact that TM3 and TM4 contain mutated p53 proteins, which are known to have an extended half-life compared to wild type proteins, reported to be 30 minutes in normal fibroblasts and 3 hours in normal human mammary epithelial cells (Delmolina et al., 1993.

The task of obtaining TM cells in the various cell cycle phases to provide suitable amounts of cells for half-life studies of P53 or p53as in G_1, S, and G_2/M was unsuccessful as reported last year. Long cell cycle times may be responsible for lack of separation of S-phase

cells from G_2/M-phase cells by centrifugal elutriation. Attempts to synchronize cells by density arrest and/or serum starvation resulted in a high fraction of non-cycling G_1 cells at the times of maximum S or G_2/M populations. The data presented in last year's progress report showed ^{3}H-thymidine uptake and flow cytometry experiments following serum starvation-induced G_1 arrest of an epidermal cell line. We emphasized last year that although it appeared by ^{3}H-thymidine uptake that the cells were synchronized, the flow cytometry data showed >57% of the cells remaining in G_1. The time points after serum replacement were 0, 29, and 35 hours. This year we address concerns that by the 29-hour timepoint the first S-phase had already occurred. The epidermal cell line used is known to have a 48 hour doubling time (Kulesz-Martin et al., 1985), so that with the time required for recovery from serum starvation, it is feasible that at 29 hours post serum replacement certain cells could still be in their first S-phase. In addition, a previous experiment utilizing ^{3}H-thymidine uptake at timepoints of 0, 8, 24, 32, and 48 hours post serum replacement showed DNA synthesis increasing through 32 hours.

We have now tried synchronizing cells utilizing aphidicolin, a G_1 cell cycle arrest agent. Five experiments were first carried out in 291 epidermal cells to optimize conditions. 291 cells were grown to approximately 60% confluency, serum starved for 72 hours after which 1 mg/ml aphidicolin was added for 24 hours. The cell monolayer was then washed and growth medium added. Aliquots of cells were removed at various time points to be analyzed by flow cytometry. Representative flow cytometry data are shown in Figure 7. Cells appear to be well arrested in G_1 following serum starvation and aphidicolin treatment (Figure 7A). Four hours after removal of aphidicolin, 33% of the cells were in S-phase and by 6 hours ~21% of the cells were in G_1, 45% were in S, and 29% in G_2. We next repeated this experiment using TM3 cells and Figure 8 shows the flow cytometry data from this. Ninety-one percent of the cells were in G_1 following serum starvation and aphidicolin treatment (Figure 8, B and C). Four hours after removing the aphidicolin the cells are beginning to move into S phase (~22%) (E). However, the number of cells in G_1 decreases only by 12% (from 71% to 59%) between 4 (E) to 12 (L) hours after removing aphidicolin. This is similar to results reported here last year obtained from serum-

starved epidermal cells in which a significant background of noncycling G_1 cells were always present. Thus, it has not been possible to determine the stability of p53 and p53as proteins at different stages of the cell cycle in the mouse mammary epithelial cells using these approaches. This may be a normal characteristic of epithelial population wherein a balance between cycling and noncycling populations is maintained. We propose to use alternative cell lines, either mouse line EL12 from Dr. D. Medina, now available in our laboratory, or human mammary line MCF7, which contains wild type p53 for susceptibility to be synchronization.

TASK 5 (Aim 3) Determination of a p53as-specific DNA binding site(s) and assay of p53as and p53 binding activities. Completion of this task was reported in the last progress report. Using cyclic amplification and selection of target DNA (CASTing) (Funk et al., 1992), both p53 and p53as were shown to bind the same DNA sequences which had certain hallmarks of the p53 consensus sequence (El-Deiry et al., 1992). In addition, a majority of the CASTing sequences contained small regions of homology to a previously reported novel p53 DNA binding sequence lacking consensus motifs (Foord et al., 1993). FASTA searches of GenBank indicated several genes contained these novel motifs in promoter or intron regions. Two such genes were the rat mdrl and the mouse thrombospondin genes, both of which have been shown to be regulated by p53 in humans (Chin et al., 1992; Dameron et al., 1994).

Table 2 shows the oligos that have been synthesized: four, ACTTG, TTGGC, INV REP, and Y-BOX, containing the novel p53 binding motifs, two, CATG MDR and CATG INV, containing consensus-like binding sequences, and a control oligo (NEG) lacking p53 binding sequences. The oligos ACTTG, TTGGC, and INV REP (an inverted repeat of TTGGC) are found in the promoter region of the mdr-1 gene or introns 2, 3, 4, and 6 of the thrombospondin gene. The Y BOX sequence contains a region so designated and known to be involved in transcriptional regulation of mdrl, which also contains our newly defined sequence TTGGC. The bold sequence shown in CATG MDR is also found in the promoter region of the rat mdr-1 gene at -199 to –180 (+1 is A in ATG). CATG INV is the same sequence as CATG MDR except that the first CATG is inverted to GTAC. Gel shift assays were performed using these oligos after

labeling with ^{32}P. Figure 9A shows the results with *in vitro* translated p53. p53 requires activation in order to bind DNA efficiently, and is expected to bind minimally or not at all in the absence of PAb421. After activation by PAb421, a supershifted band (arrow) is visible which disappears in the presence of specific competitor DNA but is unaffected by mutated DNA. Only CATG MDR probe DNA exhibited these characteristics. Figure 9B shows the results of the same assays with *in vitro* translated p53as. Since p53as is constitutively active for DNA binding, there is a visible band (small arrow) present without antibody activation which is supershifted in the presence of ApAs (large arrow). The lower band disappears in the presence of specific competitor DNA but is not competed with mutated DNA. Again, only CATG MDR probe exhibited these qualities. A positive control using the strong p53 consensus sequence hp53 DNA is shown, indicating the expected position of shifted DNA. Unprogrammed reticulocyte lysate was negative for specific binding, as was the NEG sequence (data not shown). Thus, it appears that the novel motifs alone are insufficient to bind either p53 or p53as and must require the presence of CATG (as in the original sequences retrieved by CASTing, (Miner and Kulesz-Martin, 1997) for binding to occur. The only motif of the CASTing sequences that binds, CATG MDR, is similar to the known p53 consensus sequence (El-Deiry et al., 1992). CATG MDR contains 2 CATG motifs, the second of which is preceded by 3 purines and followed by 3 pyrimidines (-189 to –180 in the mdr1 promoter), a perfect half motif consensus sequence. The sequence from -199 to -190 also contains a CATG but the surrounding nucleotides do not strictly adhere to the paradigm of upstream purines and downstream pyrimidines. It has recently been shown that sequence -247 to -126, which contains the CATG MDR sequence, is involved in the promoter function for this gene (Zhou et al., 1996). An mdrl regulatory sequence from - 189 to - 167, containing one copy of the perfect half motif consensus sequence, has been used for DNA binding assays and shown to bind two major proteins of 41 and 49 ~ from rat liver. Further examination revealed that neither protein was p53 since there was no supershifting by a p53-specific antibody. However, we have observed binding of p53 and p53as to -199 to -180 sequences which overlap the sequence examined by Zhou et al. (Zhou et al., 1996) and contains

both CATG motifs. Therefore we will test the ability of this sequence to increase transcription *in vivo* in the presence of p53. Molecular cloning of this sequence is being carried out in the laboratory in preparation for cotransfection experiments to measure transcriptional activation.

TASK 6 (Aim 2) Comparison between p53as- and p53-associated proteins. As reported last year, constructions containing full length p53 or p53as or approximately 100 amino acids of each C-terminus have been made as glutathione-S transferase (GST) fusion proteins in the pGEX-2TK expression vector. We now have produced all proteins in bacteria by induction with 200 mM isopropyl ~-D-thiogalactopyranoside (IPTG). After harvesting and lysis of the bacterial cells, the cell lysate is added to glutathione beads followed by several washes to remove unbound proteins. The TM10 nuclear extract is added to the prepared beads after which the beads are washed and associated proteins are eluted with increasing salt concentrations (200 mM, 500 mM, and 1M NaCl). Proteins from aliquots of each salt fraction are separated by SDS-PAGE and the gels are silver-stained.

Figure 10 shows the washes and 200 mM eluants from one such experiment analyzed on 12% SDS-PAGE by silver staining. Proteins from the TM10 lysate that do not associate are washed off as shown in the first 5 lanes. The 200 mM eluants usually contain few proteins as is shown in the next 5 lanes. Sizes of the protein molecular weight markers are indicated. Figure 11 shows the 500 mM and 1 M eluants from the same experiment analyzed on 12% SDS-PAGE. These fractions would be expected to contain proteins that are strongly associated with p53 and that are not associated with the negative GST control. Many proteins are associated with full length p53, p53 C-terminus, or p53as C-terminus and a lesser number associated with full length p53as. Proteins observed in similar experiments using mouse epidermal nuclear extracts are indicated by asterisks and novel mammary cell potential p53-associated proteins are indicated with +.

Figure 12 shows the same 500 mM and 1 M eluants analyzed on 7.5% SDS-PAGE. High molecular weight proteins associated with full-length p53, p53 C-terminus or p53as C-terminus are visible on this gel. Of interest is an approximately 72 kD protein that is associated with full

length p53as and a slightly higher molecular weight protein (approximately 74 kD) that is associated with the C-terminus of p53as. Proteins identified in similar experiments utilizing mouse epidermal nuclear extracts are indicated by asterisks and novel mammary cell potential p53-associated proteins are indicated with +.

Currently these same eluants are being subjected to western immunoblot analyses to rule out known p53-associated proteins including topoisomerase I, mdm2, and hsc70. The protocol will then be scaled up in order to obtain enough of selected proteins for microsequencing. Lower molecular weight molecules are easier to obtain in sufficient quantities and the HPLC tryptic digestion patterns are less complex. In addition, proteins not seen in mouse epidermal cells may be mammary cell-specific p53-associated proteins. The 72 and 74 kD molecular weight proteins predominately associated with p53as and p53as C-terminus will also be isolated for microsequencing. Scaling up will involve making nuclear extract from >4 x 10^9 TM10 cells, allowing these extracts to associate with 1 ml of prepared glutathione-GST-fusion protein bead slurry (1 mg total protein/1 ml bead), and eluting associated proteins with increasing salt concentrations. Fractions will be concentrated using an Amicon Microcon 10 filter and proteins in the concentrated fractions separated by preparative SDS -PAGE. After Coomassie Blue staining, well-resolved protein bands will be excised from the gel and sent for microsequencing. Several p53-associated proteins found in mouse epidermal cells have been isolated and microsequenced using this procedure in our laboratory.

To look for differences in p53-associated proteins in high neoplastic cells (TM4) versus low neoplastic cells (TM 10), we also will be examining associated proteins found in TM4 nuclear extracts using the same method and comparing the results to what we have observed in TM10 cells. Proteins unique to TM4 will be isolated for microsequencing in the manner described. Based on the indirect immunofluorescence data, we plan to treat cells with genistein for 3 hours to increase the levels of p53, then perform the p53 binding study. The purpose of this is two-fold: to identify p53 activating proteins which may be induced to bind to p53 by DNA damage, and to increase the amount of proteins which may be themselves induced by DNA

damage. Proteins bound to p53 in treated cells will be compared to those bound in untreated cells, and novel proteins selected for microsequencing.

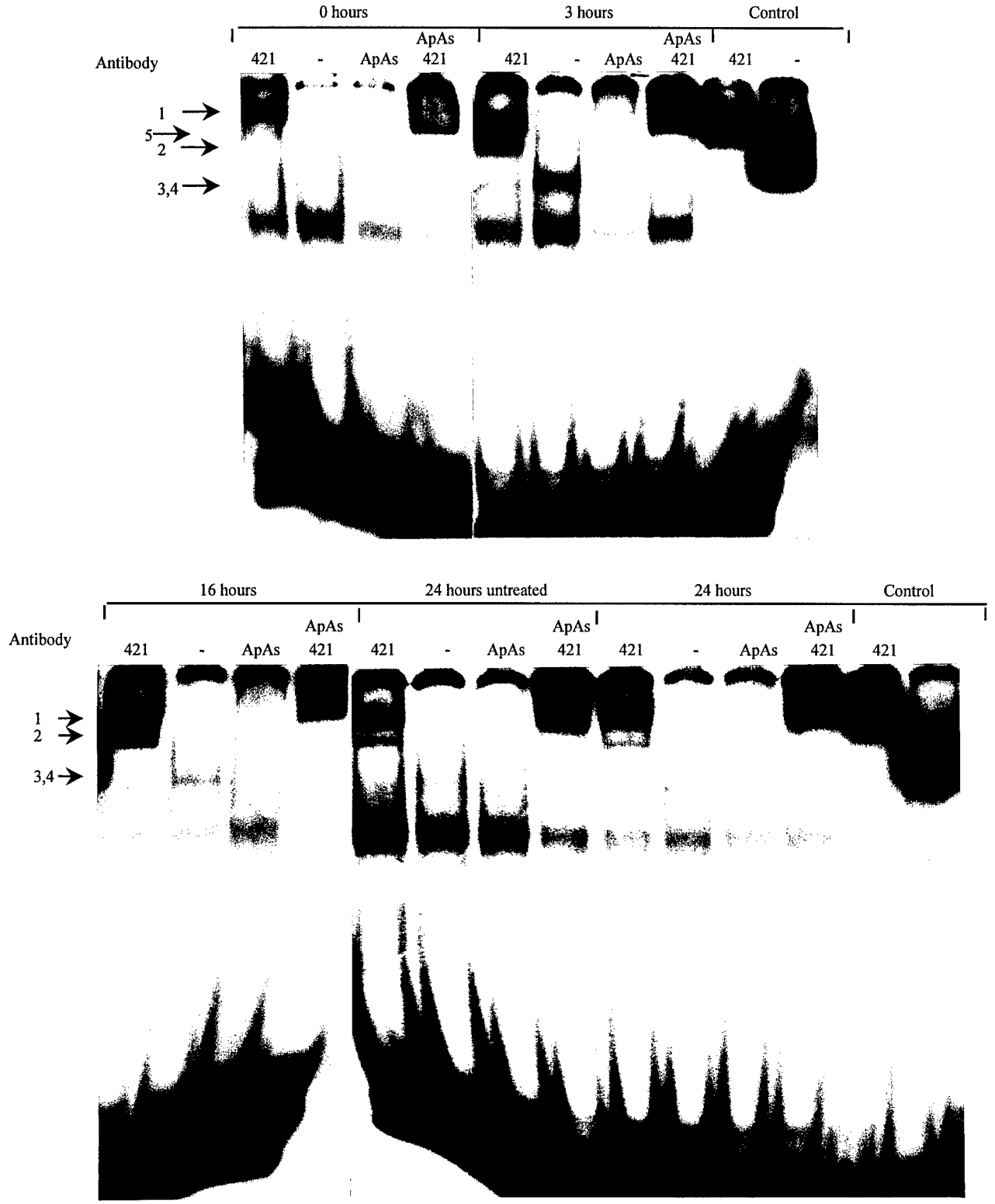

Figure 1. Gel shift assays of TM10 lysates. Binding complexes are indicated: 1, latent p53 homo-oligomer; 2, latent p53/p53as hetero-oligomer; 3, active p53as homo-oligomer; 4, active p53/p53as hetero-oligomer; 5, a putative p53-specific band visible at 3 hours in the absence of antibody or in the presence of ApAs. Control reactions contain purified p53 and p53as recombinant proteins. Lysates have been treated with 20 μM genistein unless designated untreated.

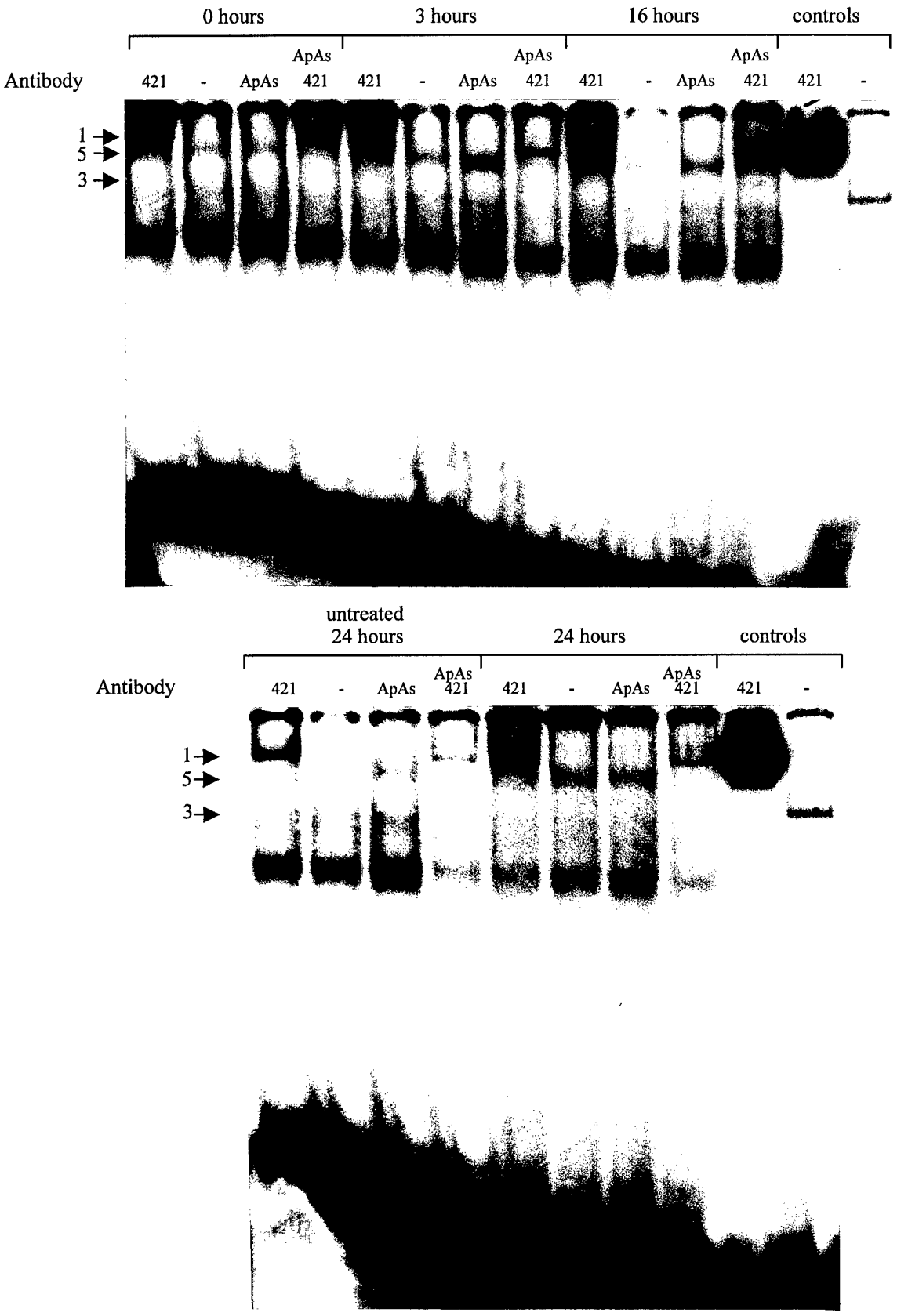

Figure 2. Gel shift assays of TM4 lysates. Binding complexes are numbered as in Figure 1: 1, latent p53 homo-oligomer; 3, active p53as homo-oligomer; 5, a putative p53-specific band. Complex 2 can be seen at 16 hours in the presence of PAb421 but is either not present or masked by complex 5 in other lanes and is not labeled for clarity. Control reactions contain purified p53 and p53as recombinant proteins. Lysates have been treated with 30 μM genistein unless designated untreated.

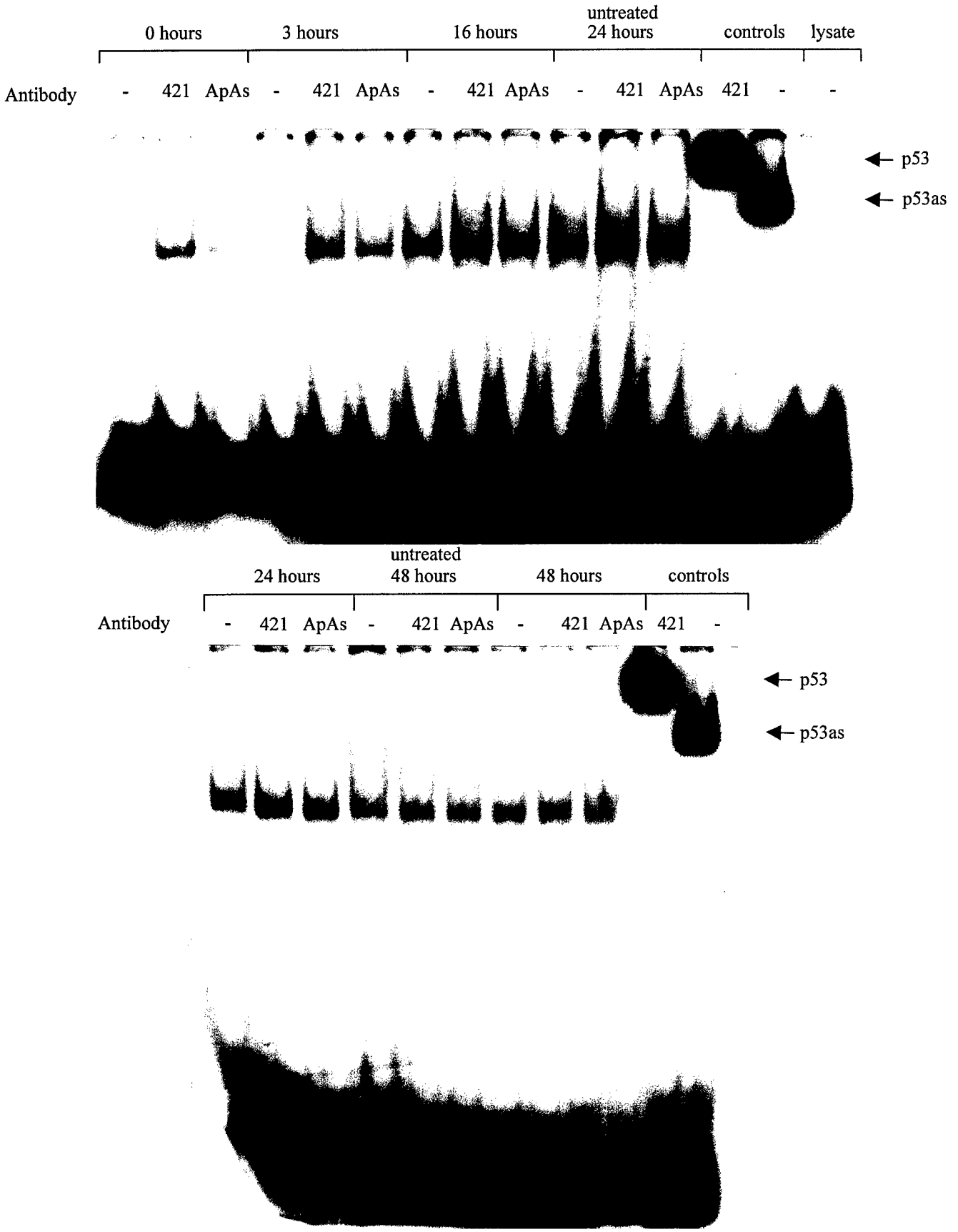

Figure 3. Gel shift assays of TM3 cell lysates. Binding complexes of controls containing purified recombinant p53 and p53as proteins are indicated. Lysates have been treated with 30 μM genistein unless designated untreated.

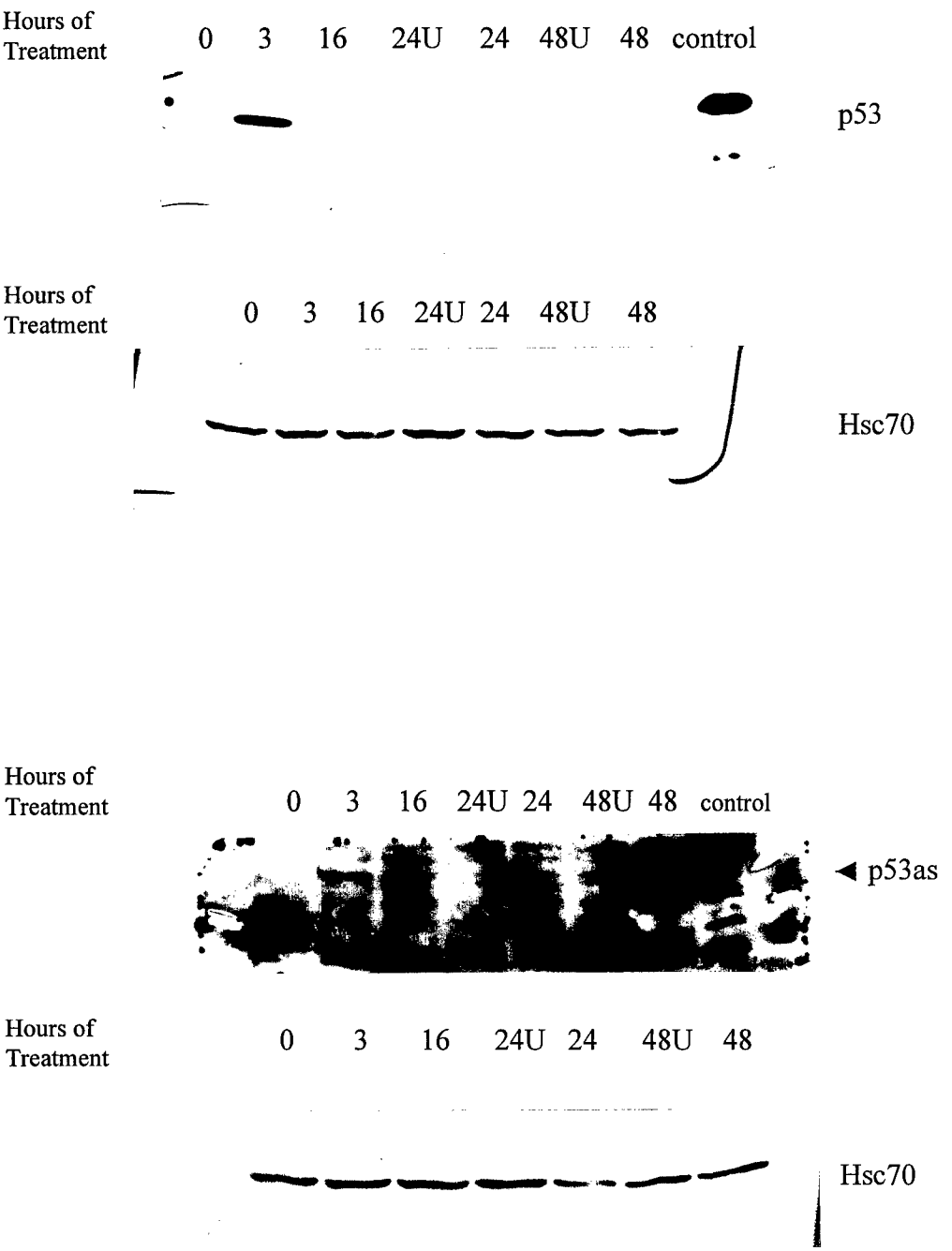

Figure 4. Western immunoblot analyses ofTM10 lysates. The same lysates used in gel shift assays shown in Figure 1 were used. The hours of treatment are indicated. Untreated lysates are designated 0, 24U, and 48U; all others are treated. Control lane contains histidine-tagged baculovirus-produced p53 or p53as which has a higher apparent molecular weight than cellular p53/p53as. Immunoblots using antibody against heat shock protein (Hsc70) are shown as loading controls.

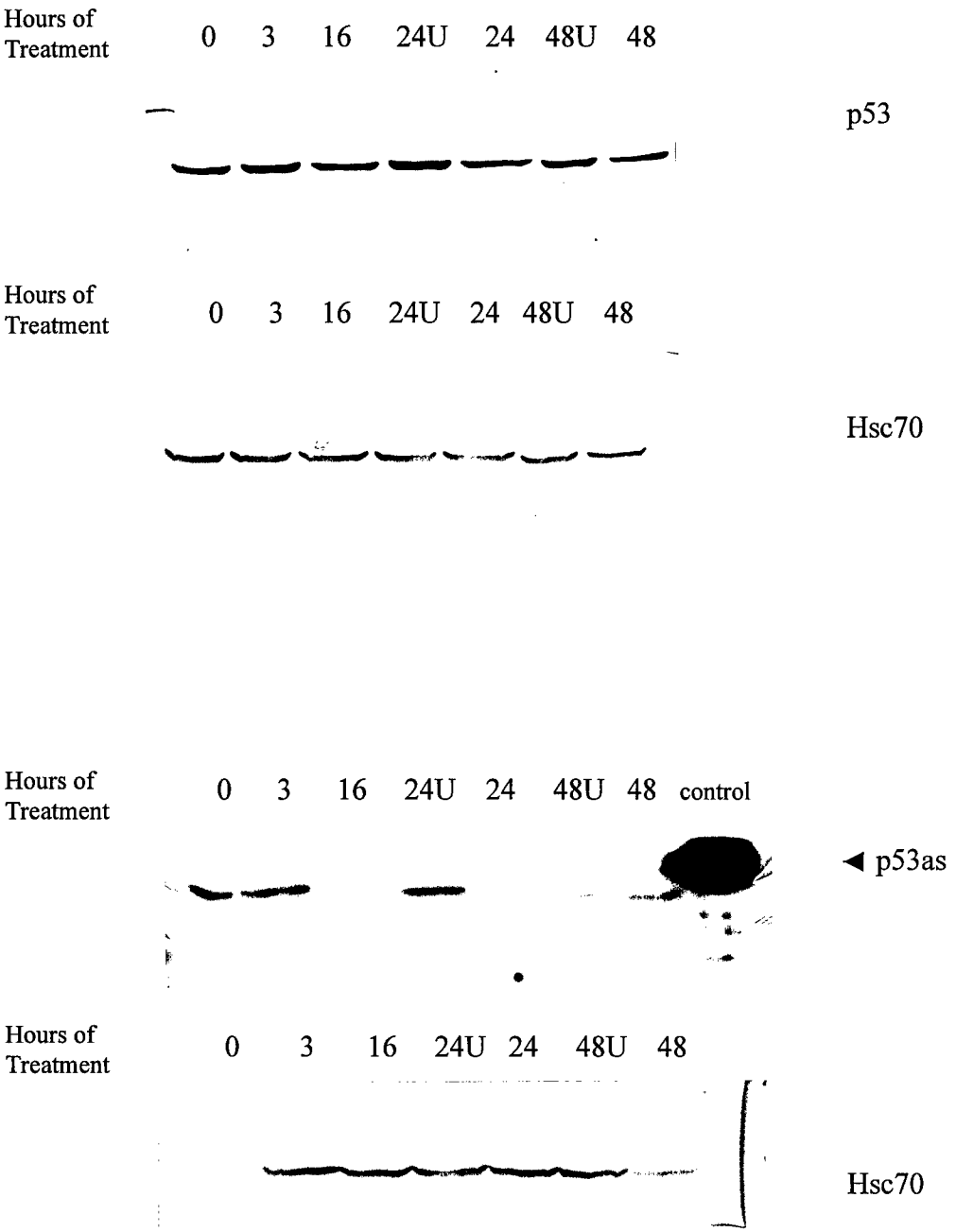

Figure 5. Western immunoblot analyses of TM4 lysates. The same lysates used in gel shift assays shown in Figure 2 were used. Conditions, times, and control lanes as in Figure 4.

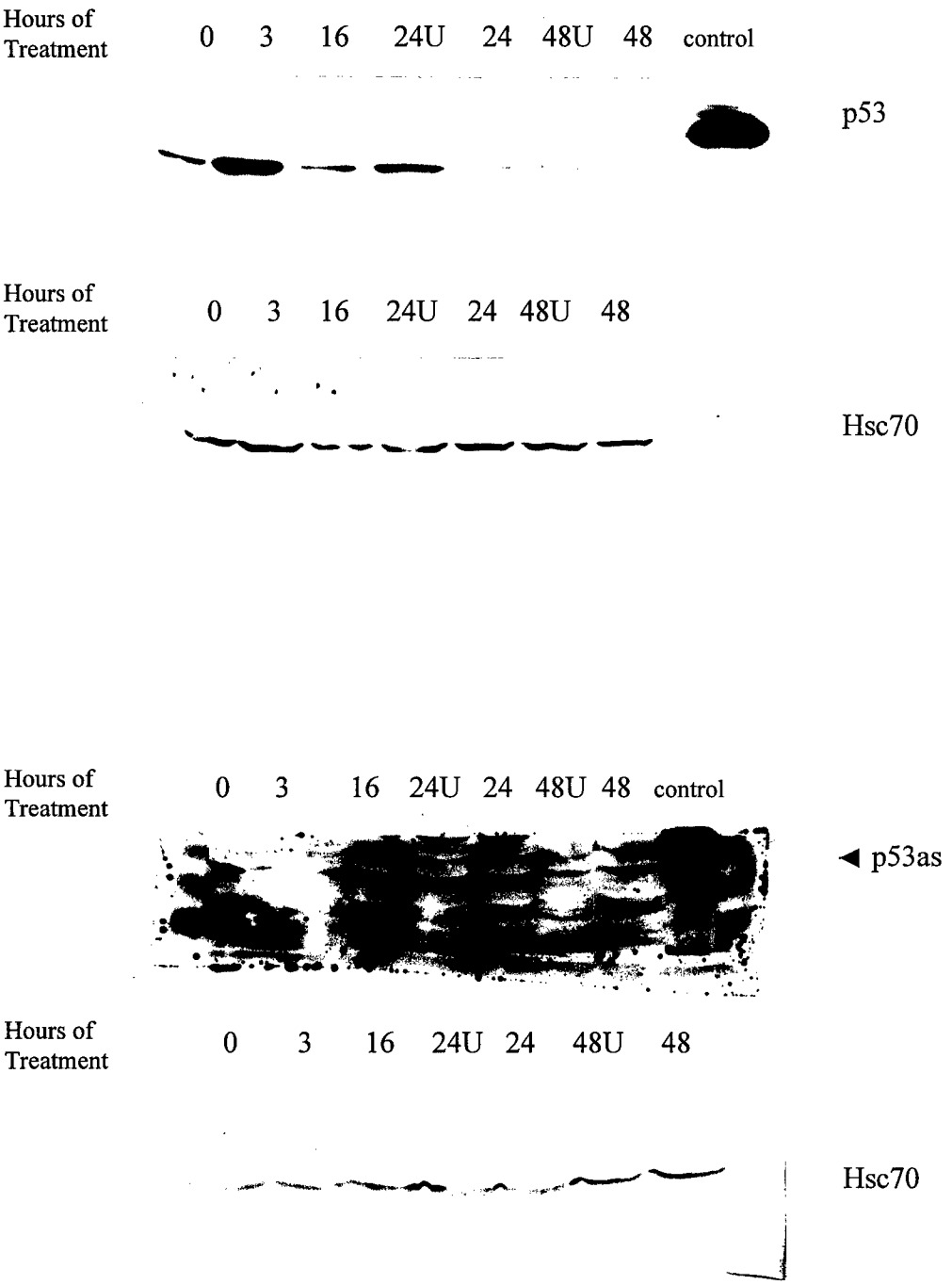

Figure 6. Western immunoblot analyses of TM3 lysates. The same lysates used in gel shift assays shown in Figure 3 were used. Conditions, times, and control lanes as in Figure 4.

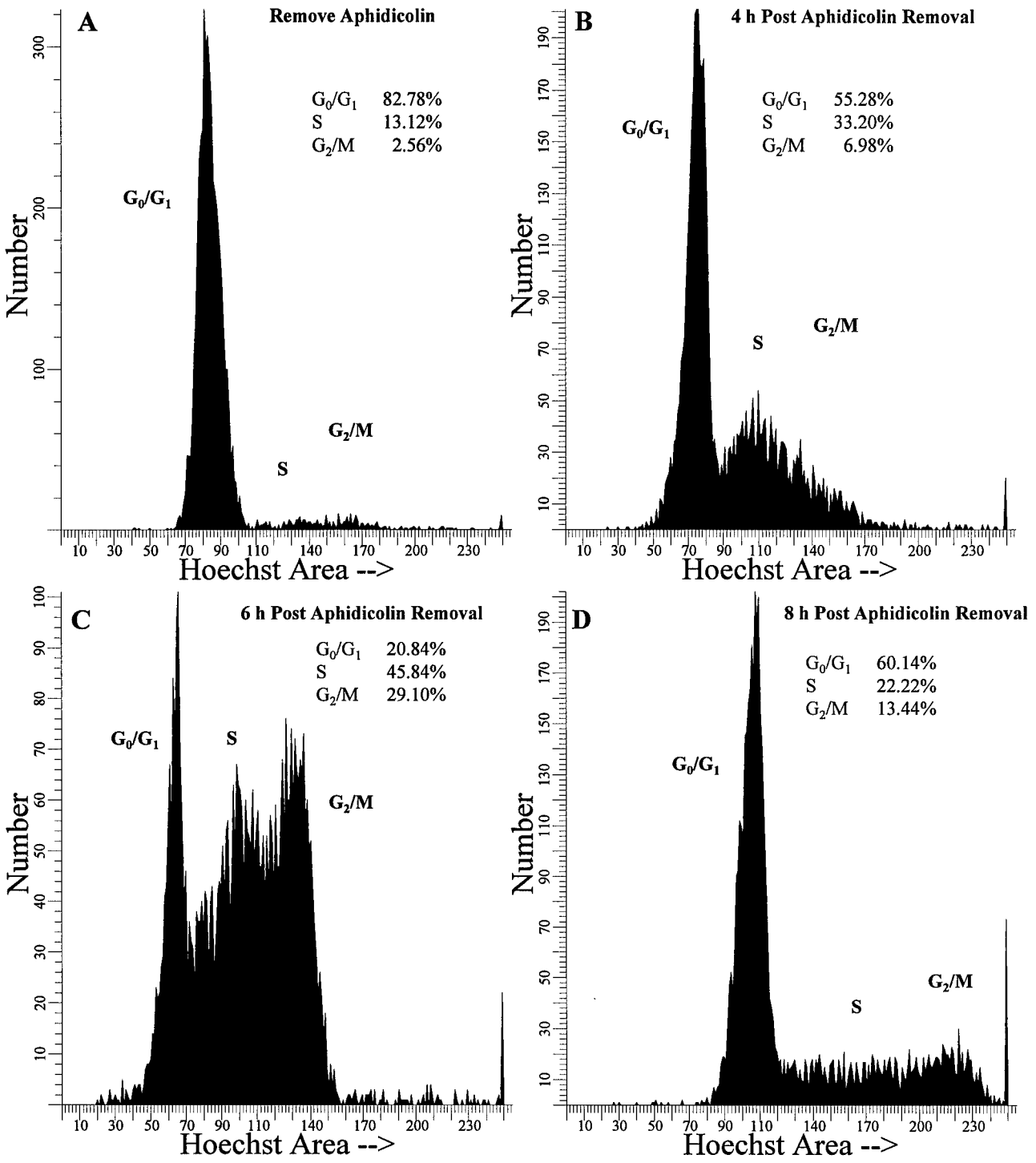

Figure 7. Flow cytometry histograms of serum-starved and aphidicolin-treated 291 cells. X-axis is Hoechst labeled DNA, Y-axis is the number of cells. Cell cycle phases are indicated.

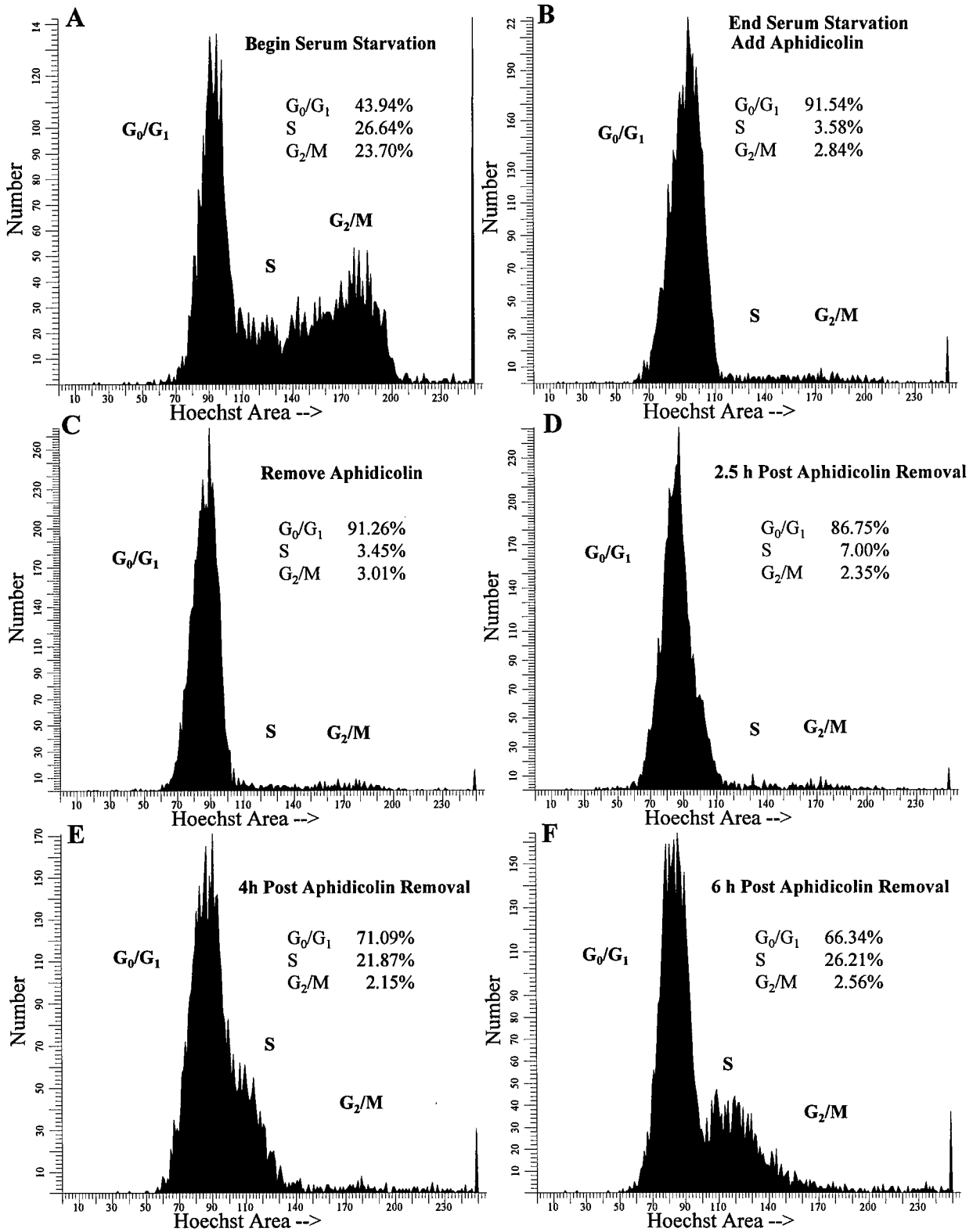

Figure 8A. Flow cytometry histograms of serum-starved and aphidicolin-treated TM3 cells X-axis is Hoechst labeled DNA, Y-axis is the number of cells. Cell cycle phases are indicated.

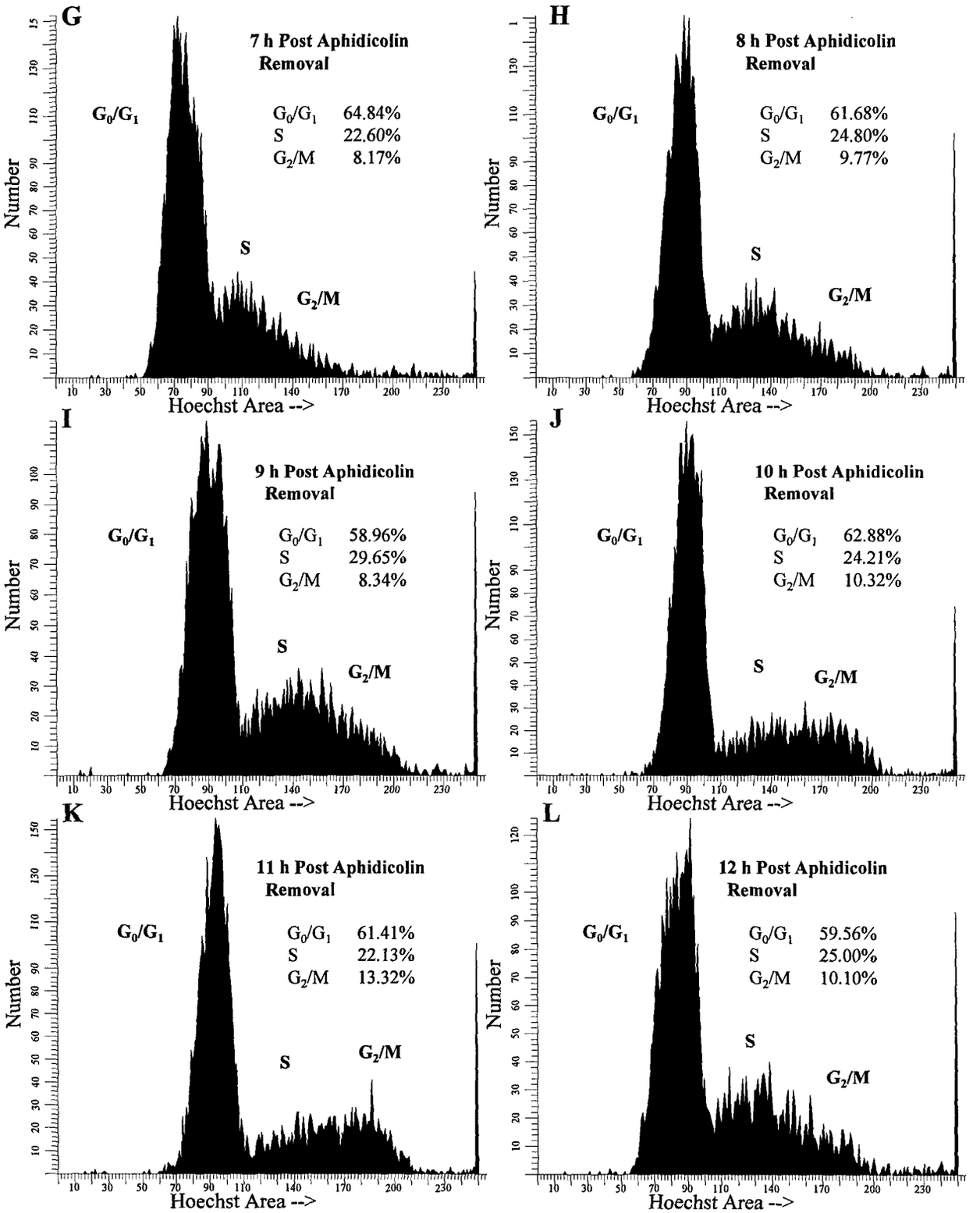

Figure 8B. Flow cytometry histograms of serum-starved and aphidicolin-treated TM3 cells. X-axis is Hoechst labeled DNA, Y-axis is the number of cells. Cell cycle phases are indicated.

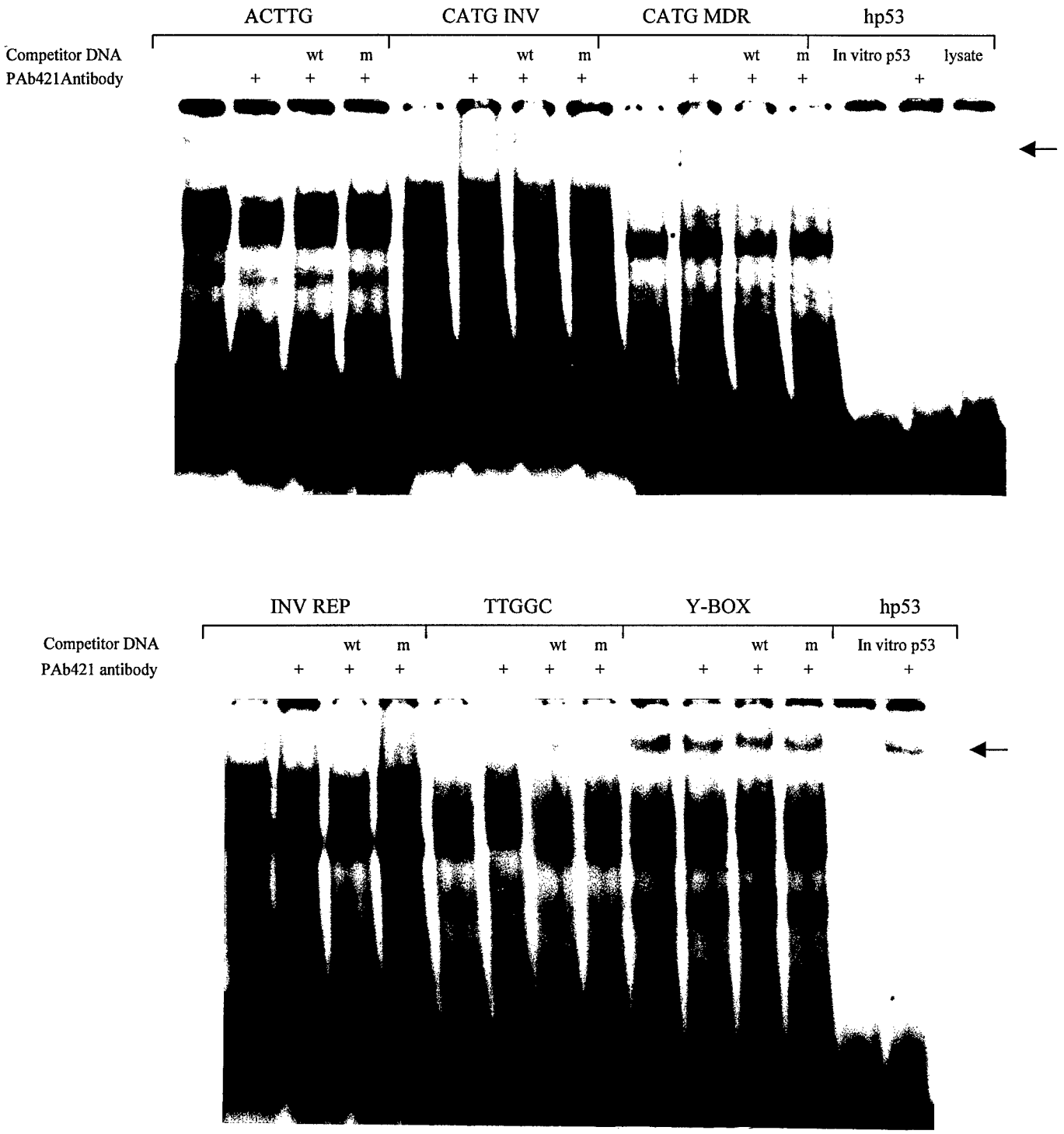

Figure 9A. Gel shift assays with *in vitro* translated p53 and segments of DNA sequences identified by CASTing as potential binding sites. The presence of antibody PAb421 is indicated. Cold competitor DNA: wt=hp53 (a p53 consensus sequence), m=mutated hp53. The arrow indicates the position of supershifted p53. P53 bound in the presence of PAb421 to hp53 is shown as a positive control. Unprogrammed reticulocyte lysate is shown as a negative control.

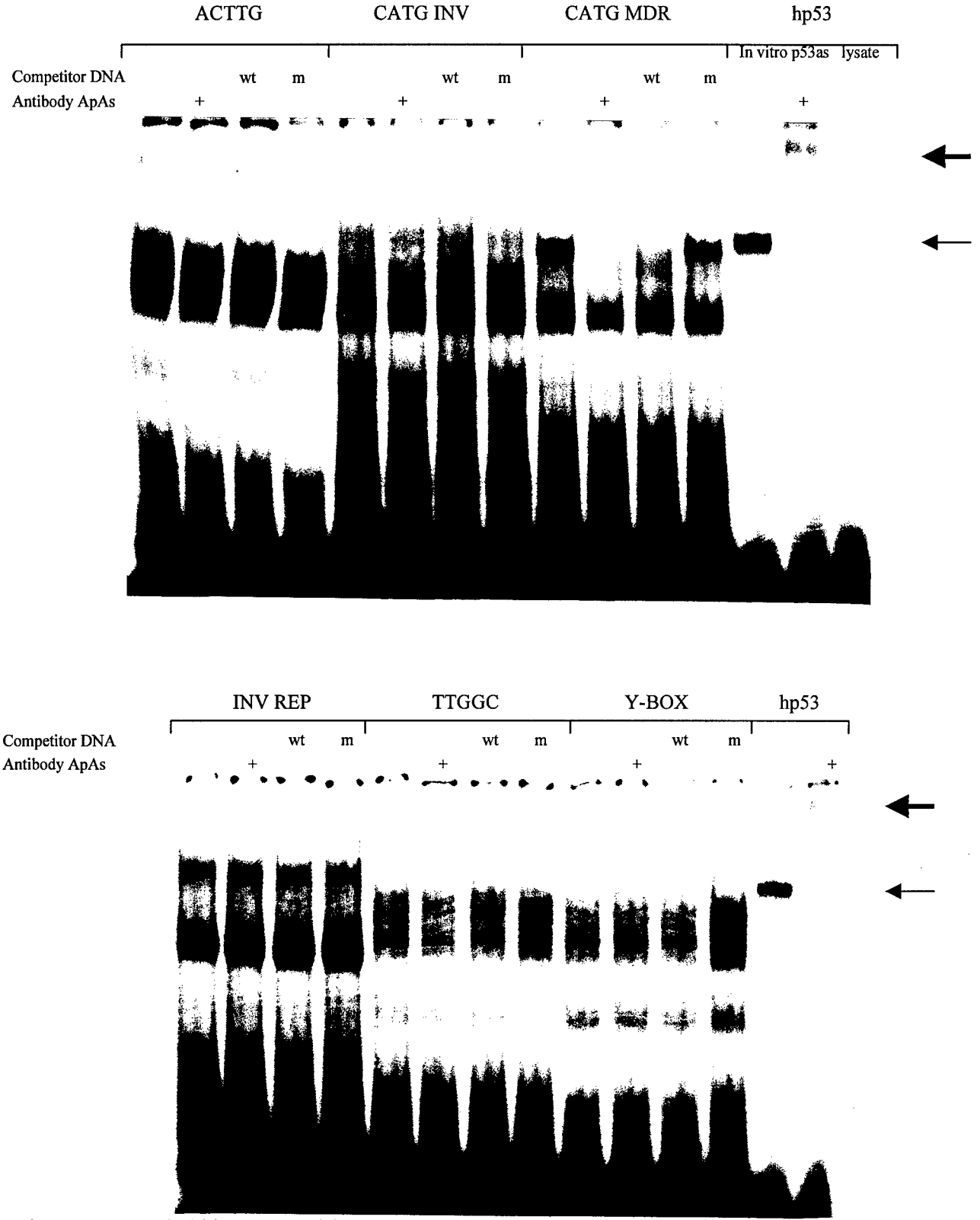

Figure 9B. Gel shift assays with *in vitro* translated p53 and segments of DNA sequences identified by CASTing as potential binding sites. The presence of antibody PAb421 is indicated. Cold competitor DNA: wt=hp53 (a p53 consensus sequence), m=mutated hp53. The arrow indicates the position of supershifted p53. The small arrow indicates p53as binding. The large arrow designates the position of supershifted p53as. p53as bound to hp53 in the presence or absence of ApAs is shown as a positive control. Unprogrammed reticulocyte lysate is shown as a negative control.

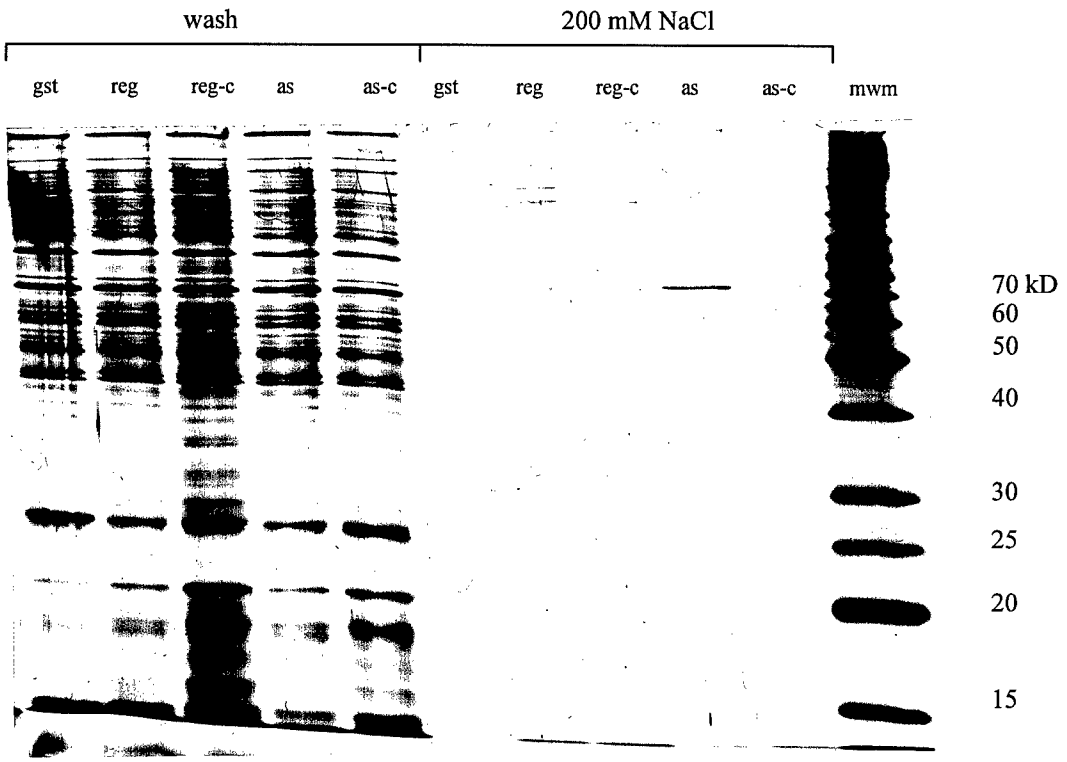

Figure 10. Silver-stained 12% polyacrylamide gel of p53 associated proteins from TM10 nuclear extracts. Wash and 200 mM eluants for gst (control), full length p53 (reg), the c-terminus of p53 (reg-c), full length p53as (as), and the c-terminus of p53as (as-c) are shown. The sizes of molecular weight markers (mwm) are indicated.

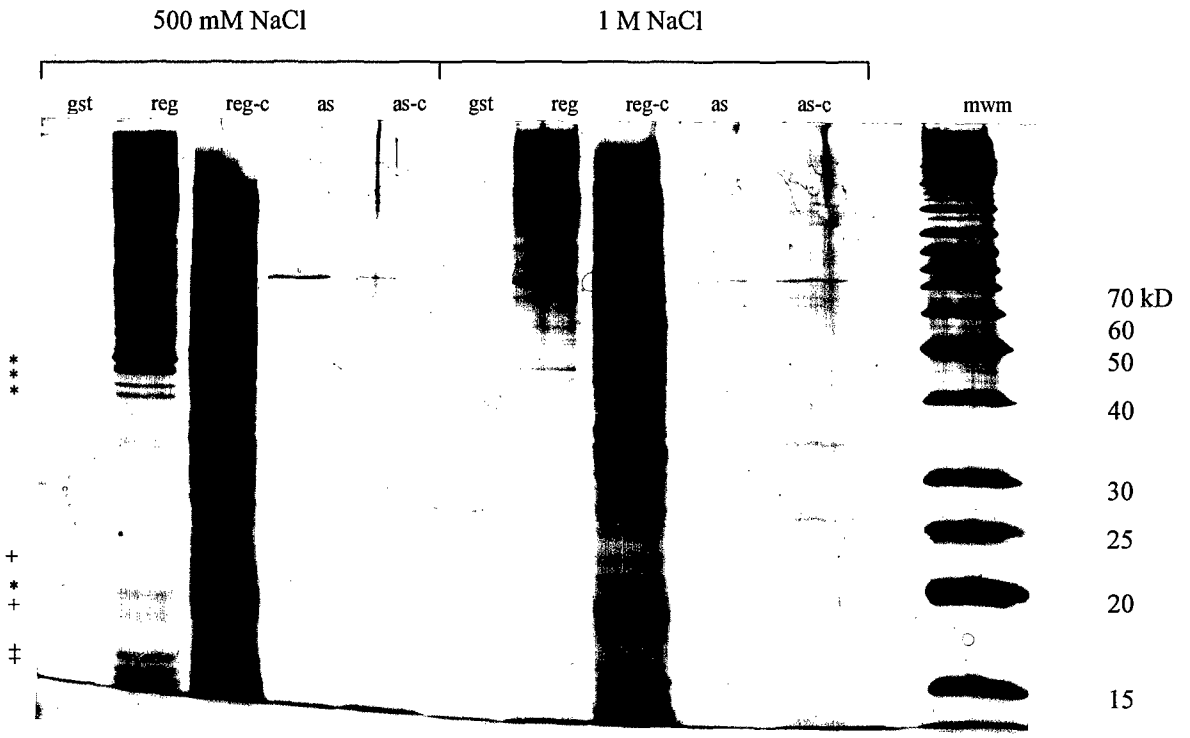

Figure 11. Silver-stained 12% polyacrylamide gel of p53-associated proteins from TM10 nuclear extracts. The 500 mM and 1 M eluants for gst (control), full length p53 (reg), the c-terminus of p53 (reg-c), full length p53as (as), and the c-terminus of p53as (as-c) are shown. The sizes of molecular weight markers (mwm) are indicated on the right. Associated proteins found in mouse epidermal cells are indicated with asterisks. Potential novel mammary-specific p53-associated proteins are indicated by a +.

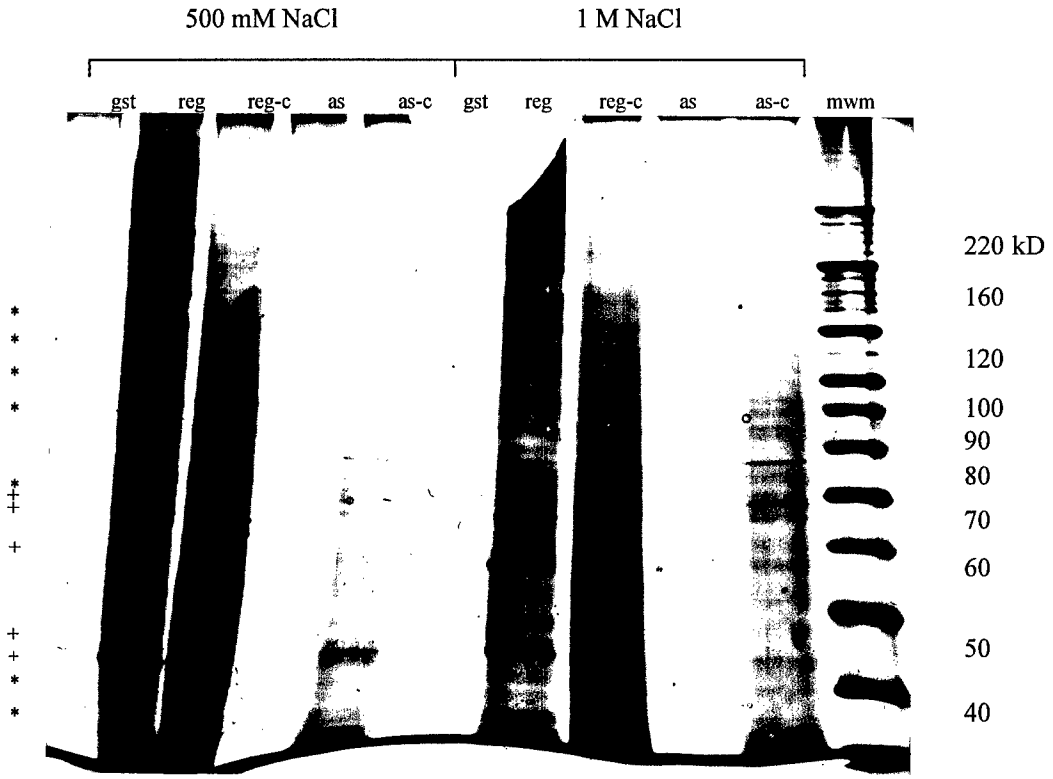

Figure 12. Silver-stained 7.5% polyacrylamide gel of p53-associated proteins from TM10 nuclear extracts. The 500 mM and 1 M eluants for gst (control), full length p53 (reg), the c-terminus of p53 (reg-c), full length p53as (as), the c-terminus of p53as (as-c) are shown. The sizes of molecular weight markers (mwm) are indicated on the right. p53-associated proteins found in mouse epidermal cells are indicated with asterisks. Potential novel mammary-specific p53-associated proteins are indicated by +.

LINE	GENOTYPE	PHENOTYPE
TM3	Mutant One allele, insertion $Ser^{233-234}$ No differing allele	Hyperplasia
TM4	Mutant One allele, deletion of amino acids 123-129 One allele, Cys --> Trp^{138}	High neoplasia
TM9	Wildtype	Low neoplasia
TM10	Not determined	Low neoplasia
TM12	Wildtype	Low neoplasia

Table 1. The tumor mammary (TM) cell lines used in this study.

NAME	SEQUENCE		
ACTTG	TCGAGAATTC	**ACTTG** TGCGCGACAG **ACTTG**	TGCGCGACAG CTCGAGTCGA
TTGGC	TCGAGAATTC	**TTGGC** TGCGCGACAG **TTGGC**	TGCGCGACAG CTCGAGTCGA
INV REP	TCGAGAATTC	TGCGCGACAG **CGGTTTTGGC**	TGCGCGACAG CTCGAGTCGA
Y BOX	TCGAGAATTC	TGCGCGACAG **CTGATTGGCT**	TGCGCGACAG CTCGAGTCGA
CATG MDR	TCGAGAATTC	**GAACATGTAGAGACATGTCT**	TGCGCGACAG CTCGAGTCGA
CATG INV	TCGAGAATTC	**GAAGTACTAGAGACATGTCT**	TGCGCGACAG CTCGAGTCGA
NEG	TCGAGAATTC	TGCGCGACAG TGCGCGACAG	TGCGCGACAG CTCGAGTCGA

Table 2. Oligos used in gel shift assays. The novel and consensus-like p53 DNA binding motifs are in bold.

CONCLUSIONS

TASK 1. Determination of p53 and p53as expression in the mouse mammary model. This task was completed at the time of our last report. The cell lines TM3, TM4, TM9, TM10, and TM12 all express p53 and p53as mRNA and protein. We chose TM3 (hyperplastic) and TM10 (low neoplastic) for immunohistochemistry analyses of tumors. Based on these studies, both tissues contain p53 and p53as. Although the p53 gene in TM10 has not been sequenced (Table 1; personal communication, D. Medina), recent indirect immunofluorescence data show that p53 proteins in TM10 cells are $PAb246^{+}$ and $PAb240^{-}$, indicating that the p53 protein conformation is wild type. The fact that a hyperplastic line, TM3, contains a mutated p53 while a neoplastic line, TM10, contains wild type p53 indicates that mutation of p53 is not an essential first step in mammary carcinogenesis, nor is it sufficient. However, it does not rule out that mutation of p53 is necessary at some point in the transition from hyperplasia to low neoplasia or to high neoplasia. This is consistent with the fact that the highly neoplastic line TM4 has a mutated p53 gene.

TASK 2. Production and purification of p53as and p53 proteins in the baculovirus system. This task has been completed with the nickel-affinity purification of histidine-tagged p53 and p53as as reported last year. These purified proteins are available for DNA binding assay controls and as an alternative to the GST-fusion protein approach to identify p53-associated proteins.

TASK 3. Determination of oligomerization properties of p53as. This task was completed in the first year and indicated that both p53 and p53as formed homo-and hetero-dimers and tetramers. Since p53as is constitutively active for DNA binding while p53 requires activation and some hetero-oligomers acquire the DNA-binding characteristics of p53, the regulation of the ratio of the two proteins may be a way to modulate the effects of the two protein forms.

TASK 4. Determination of the half-life of p53as and p53 proteins. As reported last year, the half-life of p53 (5 hours) and p53as (6 hours) in asynchronous TM3 or TM4 cells has

been determined. Obtaining TM cells in various cell cycle stages in order to determine the half life of p53/p53as in G_1, S, and G_2/M has been unsuccessful using centrifugal elutriation, serum starvation, and drug (aphidicolin)-induced G_1 arrest due to the presence of a high fraction of noncycling G_1 cells. We propose to test the feasibility of using MCF7 cells or EL12 mouse cells for synchronization and p53 protein stability studies in mammary cells.

TASK 5. Determination of a p53as-specific DNA binding site and assay of p53as and p53 binding activities. This task was completed at the time of our last progress report. Using the CASTing method [Funk, 1992 #15], both p53 and p53as were shown to bind the same DNA sequences. Oligomers of regions of these sequences containing novel DNA motifs did not bind p53 or p53as in DNA binding assays. Thus, the consensus-like regions in the CASTing sequences are necessary for binding p53 or p53as. A data bank homology search with a consensus-like sequence isolated by CASTing identified a region with 100% homology in the promoter region of the rat mdrl gene. This 20 basepair sequence (CATG MDR) binds p53 and p53as in gel shift assays and will be used in cotransfection assays to determine its effect on *in vivo* transcription. The rat multiple drug resistance gene is known to respond to p53. Although another group (Zhou et al., 1996) has evidence that p53 does not bind a sequence which overlaps a segment of CATG MDR, they apparently have not examined the entire CATG MDR sequence as we have. These experiments have identified a potential 20-basepair p53-response element in the rat mdrl gene.

Because the human MDR1 gene is also p53-responsive, we examined the promoter region in the human gene for homology to CATG MDR and found a region with 75% homology to this 20 basepair sequence. Future experiments will determine whether p53 or p53as can bind the human sequence in gel shift assays.

TASK 6. Comparison between p53as- and p53-associated proteins. This task has progressed to the stage of scaling up the GST-p53 binding assay to isolate TM10 nuclear proteins for microsequencing. Pilot studies indicate that p53as predominately associates with two proteins from this nuclear extract, 72 and 74 kD, while p53 associates with a variety of molecular

weight proteins some of which are similar in size to proteins already isolated and microsequenced from mouse epidermal cells. Six proteins have been selected as potentially mammary cell specific p53-associated proteins based on comparison with epidermal cells and will be isolated for microsequencing. The two proteins of 72 and 74 kD which appear to predominately associate with the GST-p53as fusion proteins will also be isolated and microsequenced. We are particularly interested in the fact that this molecular weight is similar to the newly reported p53 family member p73 detected in human cells (Kaghad et al., 1997). p73 has been postulated to associated with p53. That and the intriguing finding of a slowly migrating complex binding to p53 specific DNA sequences in TM4 and 10 cells leads to our proposal to test the hypothesis that p53as protein is more efficient in associating with p73. This association could be to the unique 17 amino acid C-terminus of p53as or to a region upstream of these amino acids that is still present in the C-terminal p53as GST fusion protein but sequestered in GST p53 fusion proteins due to an inhibitory C-terminal domain. In order to test the possibility that p53-associated factors are deficient in malignant cells, p53-associated proteins detectable in nuclear extracts from highly tumorigenic TM4 cells will be compared to those found in TM10 cells. Any differences could highlight p53-associated factors which, when defective, cooperate with mutant p53 to cause conversion to malignancy or malignant progression of mammary cells.

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